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Preparation and Characterisation of Homogeneous Neurotoxin Type A from *Clostridium botulinum*

Its Inhibitory Action on Neuronal Release of Acetylcholine in the Absence and Presence of β-Bungarotoxin

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1. Large-scale production and purification of complexes between *Clostridium botulinum* neurotoxin and haemagglutinin have been achieved.

2. Haemagglutinin-free neurotoxic protein of the complexes was purified to high specific neurotoxicity by affinity chromatography, on *p*-aminophenyl β-D-thiogalactopyranoside coupled to Sepharose 4B, followed by chromatography on DEAE-Sephacel.

3. The resultant neurotoxin was homogeneous on isoelectric focussing (pI = 6.3) and on dodecylsulphate/polyacrylamide gel electrophoresis under non-reducing conditions when its M_r was 1.4×10^5; after reduction two polypeptides (M_r = 9.9 and 5.5×10^4) were present.

4. On double-immunodiffusion gels, using antiserum against neurotoxin-haemagglutinin complex, the neurotoxin showed a single, sharp precipitin line that was immunologically distinct from a relatively non-toxic protein ($M_r = 1.3 \times 10^5$), which co-purifies with the neurotoxin but is removed by the ion-exchange chromatography step.

5. Application of the neurotoxin to animals *in vitro* or *in vivo* produced near complete and irreversible blockade of neurotransmission. Botulinisation of rat leg muscles reduced spontaneous transmitter release: the amplitude of miniature end-plate potentials was altered from the normal 'bell-shaped' to a skewed distribution.

6. In normal muscle, a large transient increase in frequency of the miniatures was produced by β-bungarotoxin. In contrast, with botulinised muscle the latter induced a much smaller increase in the absolute frequency; in addition, the mean amplitude was increased somewhat but the distribution remained skewed. The results show botulinisation of muscle modifies the action of β-bungarotoxin.

The most potent neuroparalytic toxins known are produced by *Clostridium botulinum* and are responsible for a serious condition, botulism, in adults and infants [1]. Of the eight immunologically distinct, but structurally related, forms of the toxin, type A is amongst the most neurotoxic and widely studied. When first isolated as an apparently single protein it was shown to have a molecular weight of 900000 and $s_{20,w}$ of 19 S [2]. This protein preparation was found to exhibit both neurotoxin and haemagglutinin activities; the latter could be removed without appreciable loss of neurotoxicity, by incubation of the complex with red blood cells in the presence of 50 mM NaCl [3]. More recently the neurotoxic moiety was separated from crystalline haemagglutinin-neurotoxin complex by DEAE-cellulose chromatography and shown to have a molecular weight of 150000 [4]; three species of haemagglutinin were resolved with molecular weights of 290000, 500000 and 900000. Purification of neurotoxin has been achieved by affinity chromatography of a pure preparation of complex on an immobilised ligand, *p*-aminophenyl β-D-thiogalactopyranoside, which is an inhibitor of haemagglutinin [5]. Use of either of these purification methods alone to prepare homogeneous neurotoxin requires pure, crystalline complex as starting material. Since repeated crystallisation is a very lengthy, laborious procedure that results in low yields, an efficient reproducible method is required for neurotoxin purification on a scale that gives the large quantities needed (see below) for immunological and neurochemical studies.

In view of the unique potency of its toxin, scientists concerned with the microbiology of *C. botulinum*, toxin detection, or studies on the neurotoxin's action should be protected by active immunisation with innocuous toxoids. Existing vaccines prepared from haemagglutinin-neurotoxin complexes are unsatisfactory in that they often induce unpleasant side-effects and have low antigenicity. In addition, immunity may be directed primarily against the more abundant haemagglutinin moieties rather than the neurotoxin component [6]. It is, therefore, believed that purified neurotoxin could provide a superior and novel vaccine. This could be used to raise immunoglobulins for therapeutic use and for development of immunoassays for the toxin in clinical specimens and foodstuffs. Finally, as the pure neurotoxin is known to inhibit specifically neurotransmitter release at peripheral [7–9] and central synapses [10], it could be an invaluable probe for nerve membrane component(s) concerned with the release process, which to date have eluded identification.

In this work procedures were, therefore, developed for the large-scale culture of the *C. botulinum* (type A), isolation of toxin complexes and efficient purification of the neurotoxin to homogeneity by affinity and ion-exchange chromatography. In addition to establishing the properties of this pro-

Definition. LD_{50}, amount of toxin that killed 50% of injected mice within 4 days.

tein, the manner in which it inhibits release of acetylcholine at the rat neuromuscular junction was examined by electrophysiological techniques, in the absence and presence of another presynaptic neurotoxin, β-bungarotoxin.

MATERIALS AND METHODS

Culture of Clostridium botulinum *and Production of Toxin*

Type A NCTC 2916 was employed; viable spores were stored at −20 °C in Robertson's meat broth. Cultures were grown at 34 °C in a medium [11] that contained proteose peptone no. 3 (20 g/l), yeast extract (10 g/l), N.Z. amine A (10 g/l) and sodium mercaptoacetate (0.5 g/l), adjusted to pH 7.3. After autoclaving, glucose was added to a final concentration of 1% (w/v). This medium was inoculated with a suspension of spores, incubated for 24 h and the resultant suspension used stepwise to seed [12] a 20-l culture. These large quantities were grown in a cabinet-enclosed fermenter, with minimal stirring and periodic flushing with O_2-free N_2 [13]. After 48 h of incubation the culture was processed for toxin purification.

Isolation of Haemagglutinin-Neurotoxin Complexes

The method used was a modification of those described previously [14, 15]. Acid precipitation of the culture was performed by pumping 1.5 M H_2SO_4 into the fermenter, with constant stirring until pH 3.5 was reached. The precipitate (55–60 g) was recovered with a continuous-flow centrifuge. This and all subsequent steps were performed in class III biological safety cabinets [13]. The pellet was homogenised and extracted with 200 ml 0.2 M sodium phosphate buffer, pH 6.0 for 1 h at 25 °C; the extract was clarified by centrifugation and the pellet re-extracted with buffer. The pooled extracts were incubated with ribonuclease (100 μg/ml; C.P. Laboratories, Herts, UK) for 3 h at 34 °C.

Following precipitation with 60% saturated ammonium sulphate, the sedimented toxin was dissolved in 50–100 ml 0.05 M sodium citrate buffer, pH 5.5, and dialysed against this solution at 4 °C. Much of the brown pigment present at this stage was removed by adding a half volume of swollen DEAE-Sephadex A-50 and allowing it to stand for several hours at 25 °C followed by removal of the resin by centrifugation. The clear solution was loaded onto a column (90 × 8 cm) of DEAE-Sephacel equilibrated with the latter buffer; toxin was eluted in the void volume and fractions having a 260/280 nm absorbance ratio of 0.54–0.58 were pooled. After precipitation with ammonium sulphate as above, the protein was dissolved in 0.03 M sodium phosphate buffer, pH 6.8 and dialyzed against the latter.

Removal of Haemagglutinin by Affinity Chromatography

p-Aminophenyl β-D-thiogalactopyranoside, an irreversible haemagglutinin inhibitor, was coupled to CH-Sepharose 4B, as described by Moberg and Sugiyama [5]. The extent of coupling was over 73% as determined by A_{255} of the reaction mixture supernatant; this represented approximately 10 μmol of ligand/ml gel. The slurry was washed with 100 ml each of 50 mM sodium phosphate buffer containing 1 M NaCl at pH 7.9 and then pH 6.3 before rinsing with water. Less than 3% of the coupled ligand was lost during storage at 4 °C in aqueous solution containing preservative.

The neurotoxin-haemagglutinin complex in 50 mM sodium phosphate, pH 6.3 (≈ 7 mg/ml) was reacted in a batch with the gel for 2 h at 25 °C with gentle stirring. The resin was then packed into a 10-ml column and the solution was recycled through the column by pumping for 0.5 h. After this time the fluid was collected from the column; the resin was washed with 50 ml loading buffer and neurotoxin was eluted, with 0.1 M sodium phosphate pH 7.9 containing 1 M NaCl, from the immobilised haemagglutinin. Protein concentration of all samples was measured colorimetrically [16] using bovine serum albumin as standard.

Isolation of Neurotoxin by Anion-Exchange Chromatography

The eluate from the affinity column, or in some experiments a preparation of complex, in 150 mM Tris/HCl pH 7.9, was loaded onto a DEAE-Sephacel column and washed with two column volumes of the latter buffer before applying 300 ml of a linear NaCl gradient (0–0.5 M) in the same medium.

Analytical Methods

Toxicity Test. Samples, serially diluted with sterile 0.2% (w/v) gelatin 0.07 M sodium phosphate buffer, pH 6.5, were assayed for toxicity by intraperitoneal injection into groups of 4 mice and the concentration that killed half of the animals within 4 days was taken as LD_{50}.

Assay of Haemagglutinin Activity. This was determined in 0.05 ml of serially diluted samples using an equal volume of 0.5% suspension of chick red blood cells, as described previously [17]. The lowest dilution (μg protein/ml) that gave a positive reaction after 2 h at 4 °C was determined.

Electrophoresis. Polyacrylamide slab gels (4–30%; PAA 4/30 Pharmacia) were used [18]. For sodium dodecyl sulphate gels, samples were heated at 60 °C for 10 min in 2.5% dodecyl sulphate/10 mM Tris-HCl pH 8/1 mM EDTA/6% sucrose with and without 5% 2-mercaptoethanol; the electrophoresis buffer was 40 mM Tris/20 mM sodium acetate, pH 7.4, containing 2 mM EDTA and 0.2% sodium dodecyl sulphate. Electrophoresis under native conditions was performed in 90 mM Tris/80 mM boric acid pH 8.2 containing 3 mM EDTA. All gels were prerun at 100 V for 1 h prior to sample application. Dodecyl sulphate gels were run at 150 V at 4 °C until the tracking dye (bromophenol blue) migrated off the end; voltage was then lowered to 100 V for 2 h. Native gels were run at 150 V for 18 h at 4 °C. Gels were fixed and washed overnight with 25% isopropanol/10% acetic acid. Protein was stained with 0.04% Coomassie blue G-250 in 3.5% perchloric acid; destaining was in 10% acetic acid. For molecular weight determination, standard proteins (thyroglobulin, ferritin, albumin, catalase and lactate dehydrogenase) were run simultaneously in a track of the same slab.

Ouchterlony Double Immunodiffusion

Horse antiserum against acid-precipitated type A toxin complex was used. Samples (5 μl) of toxin or antibody were placed into wells (2.5 mm diameter) cut in 1% Agarose gel in 0.1 M phosphate buffer pH 7.0, cast on glass plate (50 × 50 × 2 mm). After allowing the sample to diffuse at 4 °C for 20 h, the gels were stained for 10 min with 0.05% Coomassie blue R-250 in 40% methanol/10% acetic acid; destaining was in the latter solvent.

Electrophysiological Recording

In initial experiments, sartorius muscle together with its nerve was dissected from frog and placed in well oxygenated frog Ringer solution. The nerve was stimulated supramaximally at 7 stimuli/min (0.1 ms pulse width) and tension was recorded with a Grass transducer. The toxin was added in 0.2 ml frog Ringer containing 0.05% gelatin. Subcutaneous injections of 60 pg pure neurotoxin were given into the hindleg of Sprague-Dawley rats (150 g) and the extensor digitorum longus muscle was removed 1–3 days later [19]. The muscle was transferred quickly to a recording chamber perfused with oxygenated (95% O_2 + 5% CO_2) Krebs solution: intracellular voltage recording was carried out at 22–25 °C with microelectrodes filled with 3 M KCl, as described previously [20]. End-plates were located by finding miniature end-plate potentials with fast rise time (< 1 ms). In botulinised muscle there was a wide scatter in these rise times [19], and recordings were made only if at least some of the miniatures had fast rise times. Voltage recordings were stored on magnetic tape, and analysed subsequently by computer. All measured miniatures were inspected visually to eliminate artifacts. β-Bungarotoxin, purified to homogeneity by ion-exchange chromatography and preparative isoelectric focussing [21], was applied in the bath and during this stage perfusion was arrested. Values quoted are means ± standard error of the mean, while Student's *t*-test (one-tailed) was used to test significance.

RESULTS

Purification and Characterization of Neurotoxin-Haemagglutinin Complexes

For controlled and reproducible production of crude toxin, *Clostridium botulinum* type A was grown in a stirred fermenter; the amount of toxin present reached a peak of $0.5-1.0\times10^6$ mouse LD_{50} units ml culture after 24 h and remained at this level even during cell lysis (Fig.1). After acid precipitation most (> 90%) of the neurotoxin-haemagglutinin complexes could be extracted at pH 6.0–6.5 with a resultant 3.3-fold purification (Table 1). Following a digestion with ribonuclease, separation of protein from nucleotides and nucleic acids by column chromatography on DEAE-Sephadex was facilitated by a prior batchwise treatment with the gel; this removed some neurotoxin (23%) but resulted in removal of most of the brown pigment present which, otherwise, caused blockage of ion-exchange columns used subsequently. The neurotoxic activity was recovered from the DEAE-Sephadex column in high yield (96%) in the void volume. It had a specific neurotoxicity of 25 mouse LD_{50} units/ng protein, which represents a 15.6-fold purification relative to the acid-fractionated culture (Table 1); this material also possessed haemagglutinin activity, which was enriched 30-fold during the purification.

The material purified as described in Table 1 was analysed by gradient pore electrophoresis under native conditions at pH 8.2; it showed five distinct bands with molecular weights of 1.1×10^6, 8.5×10^5, 7.7×10^5, 1.3×10^5 and 9.7×10^4 together with a weak diffuse band in the region of $(3.5-5.0)\times10^5$ (Fig. 2). Several peaks could also be separated by gel filtration on Sephacryl S-300; the major one corresponded in size and approached the purity of crystalline neurotoxin-haemagglutinin complex. In Ouchterlony double-diffusion gel, using antiserum prepared against partially purified haemagglutinin-neurotoxin complex, the material from stage 8 (Table 1) gave two precipitin lines; the inner line was somewhat broad and seemed possibly to be a doublet (Fig. 3).

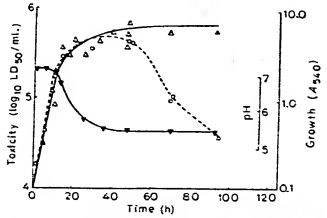

Fig.1. *Growth of* Clostridium botulinum *type A and toxin production.* Fermentation of strain NCTC 2916 was performed in a stirred fermenter at 34 °C as described in Materials and Methods. Concentration of toxin (Δ) was measured by lethality tests in mice. Bacterial growth was assessed by measurement of A_{540} (○) and confirmed by an independent assay of the number of bacteria/ml. pH (▼) of the culture was also monitored

Table 1. *Purification of* C. botulinum *type A haemagglutinin-neurotoxin complexes*
Haemagglutination activity is given as the minimum concentration of protein (μg ml) required to give a positive haemagglutination

Stage	Procedure	Protein	Haem-agglutinin activity	Total toxicity	Specific neuro-toxicity	Recovery of toxicity	
						per stage	overall
		mg	μg ml	mouse LD_{50} units	LD_{50} ng protein	%	
1	Whole culture (20 l)	–	–	1.0×10^{10}	–	100	100
2	Precipitation at pH 3.5, adjusted with 1.5 M H_2SO_4	4600	203	7.5×10^9	1.6	75	75
3	Extraction with 0.2 M phosphate buffer at pH 6.0	1300	25.3	6.8×10^9	5.2	91	68
4	Ribonuclease treatment (100 μg ml, 34 °C, 3 h)	1300	–	6.8×10^9	5.2	100	68
5	Precipitation at 60% saturation $(NH_4)_2SO_4$ (at 25 °C)	–	–	6.5×10^9	–	96	65
6	DEAE-Sephadex A-50 batch preabsorption	860	35.4	5.0×10^9	5.5	77	50
7	DEAE-Sephadex A-50 ion-exchange chromatography at pH 5.5	195	–	4.8×10^9	25.0	96	48
8	Precipitation at 60% saturation $(NH_4)_2SO_4$ (at 25 °C)	195	6.7	4.8×10^9	25.0	100	48

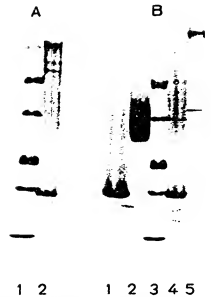

Fig. 2. *Gradient-pore gel electrophoresis of toxin samples at various stages of purification.* This was performed in 4–30% acrylamide slab gels in 90 mM Tris-80 mM boric acid pH 8.2/3 mM EDTA at 150 V for 18 h at 4°C; they were fixed and then stained with Coomassie G-250. (A) Track 1, marker proteins: thyroglobulin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin (in order of decreasing size from top of gel). Track 2, the preparation of toxin complex used for the purification of neurotoxin. (B) Tracks 1 and 2, second and first peaks after DEAE-Sephacel chromatography (Fig. 6) of material purified by affinity chromatography. Track 3, standard molecular weight proteins as in (A). Tracks 4 and 5, eluate and breakthrough from affinity column (Fig. 4)

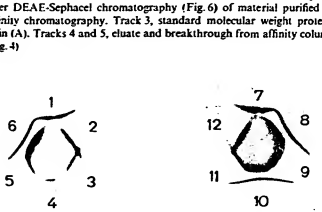

Fig. 3. *Ouchterlony double-immunodiffusion gels of purified proteins from* Clostridium botulinum. The centre wells of the agarose gels contained horse antiserum against crude type A toxin complex; protein samples were placed in outer wells and allowed to diffuse for 20 h at 4°C before staining the protein with Coomassie R-250. Wells 6, 8 and 10, haemagglutinin-neurotoxin complex; 9, eluate from affinity column (Fig. 4); 2 and 4 first and second peaks, respectively, from DEAE-Sephacel chromatography (Fig. 6) of the material from affinity column; 12, first peak from chromatography of haemagglutinin-neurotoxin complex on DEAE-Sephacel (Table 2 procedure B); 1 and 7 second peak from the latter; 3, 5 and 11 third peak from DEAE-Sephacel. The lines from wells 3 and 4 coalesced

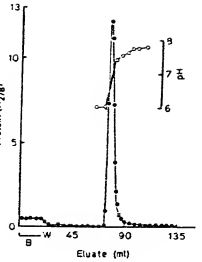

Fig. 4. *Affinity chromatography of haemagglutinin-neurotoxin complexes on p-aminophenyl β-D-thiogalactopyranoside–Sepharose 4B.* The sample was reacted with the gel in 50 mM phosphate buffer pH 6.3 for a total of 2.5 h at 25°C, breakthrough (B) collected, washed with equilibrating buffer (W), and then eluted with 0.1 M phosphate buffer pH 7.9 1 M NaCl. pH (○) and A_{278} (●) of the fractions were measured

Isolation and Identification of Haemagglutinin-Free Neurotoxin

Material Purified by Affinity Chromatography. This was achieved by the use of *p*-aminophenyl β-D-thiogalactopyranoside [5] covalently attached to Sepharose for binding the haemagglutinin moiety of the complex; the neurotoxin was dissociated and eluted by medium of higher ionic strength and with more alkaline pH, leaving the haemagglutinin attached to the resin. After application of complex (from stage 8, Table 1) to the affinity gel 97% of the neurotoxicity was absorbed; on washing with equilibration buffer the remaining 3% was recovered. The majority of the neurotoxin (76%) was eluted as a single protein peak (Fig. 4). It had a specific neurotoxicity of 44 mouse LD_{50} units/ng of protein and lacked detectable haemagglutinination activity (Table 2). In native gel electrophoresis this material showed one diffuse band (in the molecular weight range $3\times10^5-5\times10^5$) together with a sharp one with a molecular size of about 1.3×10^5 (Fig. 2). This neurotoxin gave a broad precipitin line (possibly a doublet) on a double-immunodiffusion gel, that showed partial identity with the inner band observed with complex (Fig. 3). In the case of the neurotoxin preparation, the notable absence of the outer line suggests (together with other evidence given below) that this may be due to removal of haemagglutinin by the affinity chromatography step. In dodecyl sulphate gel electrophoresis the preparation showed only two components of molecular weights of 1.4×10^5 and 1.25×10^5 compared to the multiple bands present in the purified complex (Fig. 5), under non-reducing conditions.

Two Components Separated by Sequential Affinity and Ion-Exchange Chromatography. The two proteins present in the material purified by affinity chromatography (Fig. 4) can be resolved by chromatography on DEAE-Sephacel (Fig. 6). Neurotoxin was recovered in the column void volume and had a specific neurotoxicity of 83 mouse LD_{50} units/ng protein; this respresented an overall purification of 52-fold relative to the acid-precipitated culture extract (Tables 1 and 2). In addition, a broad protein peak was subsequently eluted (Fig. 6) but this had a very much lower (1383-fold) specific neurotoxicity (Table 2) and contained a negligible amount of the applied toxicity. The neurotoxin peak exhibited a broad band (as before) under native conditions on gradient pore electrophoresis (Fig. 2) but showed a single, sharp band on dodecyl sulphate gel electrophoresis

Table 2. *Purification of* C. botulinum *type A neurotoxin components*
Haemagglutinin activity is given as the minimum concentration of protein (μg/ml) required to give a positive haemagglutination

Procedure	Protein	Haem-agglutinin activity	Total toxicity	Specific neuro-toxicity	Recovery of toxicity	
					per stage	overall
	mg	μg/ml	mouse LD_{50} units	LD_{50}/ng protein	%	
A. Neurotoxin-haemagglutinin complex purified to stage 8, Table 1	108.0	6.7	2.7×10^9	25	100	–
Affinity chromatography eluate	44.8	0	2.0×10^9	44	76	37
DEAE-Sephacel ion-exchange chromatography						
a) First peak	14.3	0	1.2×10^9	83	60	23
b) Second peak	23.7*	0	1.4×10^8	0.06	0.1	< 0.1
B. Neurotoxin-haemagglutinin complex from stage 8, Table 1	44.0	6.7	1.1×10^9	25	100	–
DEAE-Sephacel chromatography						
a) First peak	5.4	0	0.2×10^9	37	18.8	9.0
b) Second peak	10.1	4.0	1.2×10^6	0.01	0.11	–
c) Third peak	3.3	0	3.0×10^5	0.01	0.02	–

* This presents only the central fractions of the second peak (Fig. 6).

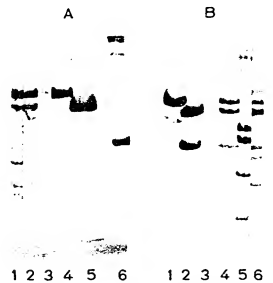

Fig. 5. *Dodecyl sulphate polyacrylamide gel electrophoresis of samples at the different stages of toxin purification.* Gels were run without (A) and with (B) 5% 2-mercaptoethanol. Amounts of protein in the samples applied to the gels ranged from 20 μg to 70 μg per track. The gels were fixed, and stained as described in Materials and Methods. (A) Gels run under non-reducing conditions; track 1 preparation of haemagglutinin-neurotoxin complexes. Tracks 2 and 3 eluate and breakthrough, respectively, from affinity column (Fig. 4). Tracks 4 and 5 first and second peaks obtained from DEAE-Sephacel chromatography (Fig. 6) of material purified by affinity chromatography. Track 6 standard proteins as noted below. (B) Gels run under reducing conditions; tracks 1 and 2 second and first peaks, respectively, obtained after DEAE-Sephacel chromatography of material purified by affinity chromatography. Tracks 3 and 4 breakthrough and eluate from affinity column. Track 5 standard proteins [thyroglobulin, ferritin (half unit), albumin, catalase, lactate dehydrogenase, ferritin, in order of decreasing size from top of gel]. Track 6 haemagglutinin-neurotoxin complex

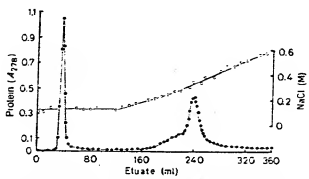

Fig. 6. *DEAE-Sephacel chromatography of toxin purified by affinity chromatography.* Haemagglutinin-free toxin (44.8 mg) eluted from the affinity gel (Fig. 4) was dialyzed against 0.15 M Tris HCl, pH 7.9, and applied to the ion-exchange column (50 × 1 cm). After washing with two bed volumes, a linear NaCl gradient (0–0.5 M) in the same buffer was applied. A_{278} (●) and chloride concentration (○) were monitored, in the latter case by a $Hg(CNS)_2$ colorimetric method

under non-reducing conditions, with a molecular weight of 1.4×10^5 (Fig. 5); this indicates that under the conditions of the native gel the neurotoxin may self-aggregate. On isoelectric focussing in polyacrylamide gel, using a broad pH (3.5–9.0) gradient, the purified neurotoxin gave a single protein band with a pI of 6.3. Thus, the neurotoxin is homogeneous with respect to size and charge.

The neurotoxin contained about equal amounts of two polypeptides with molecular weights of 9.9×10^4 and 5.5×10^4 (Fig. 5). On the other hand, the second component eluted (Fig. 6) gave molecular weights of 1.3×10^5 and 1.25×10^5 on native and dodecyl sulphate gel electrophoresis, respectively (Fig. 2 and 5) but this slight difference is probably due to variations in the techniques used; minor components were obvious also. On immunodiffusion gels the pure neurotoxin gave a single precipitin line that was antigenically different from the band seen with the 1.3×10^5 molecular weight component (Fig. 3). This is consistent with the partial identity seen between the material purified on affinity resin only and each of the peaks from the subsequent DEAE-Sephacel chromatography.

Neurotoxin and Haemagglutinin Moieties Purified by DEAE-Sephacel Chromatography

For comparison an alternative method (procedure B, Table 2) was tested for the isolation of neurotoxin from the complex; in contrast to procedure A (Table 2) this allows the haemagglutinin to be recovered. The material from stage 8 (Table 1) of the purification scheme for neurotoxin-haemag-

Table 3. *Effects of botulinisation of rat muscle on amplitude and frequency of miniature end-plate potentials measured in the absence and presence of β-bungarotoxin*

Recordings were made in extensor digitorum longus muscle of rats with and without (control) injection with 60 pg botulinum neurotoxin. All the values are means ± standard error of means from the number of fibres shown in the first figure in parentheses, the second figure in parentheses represents the number of animals used. Coefficient of variation = standard deviation mean. Skewness = mean-median/standard deviation. Mean resting membrane potential in these experiments was 67.3 ± 0.6 mV (S.D. = 5.7 mV). At each end plate an average of 184 miniature end-plate potentials were measured to calculate their mean amplitude

	Without β-bungarotoxin				After (14–45 min) addition of β-bungarotoxin (0.14 μM)			
	miniature end-plate potential		coefficient of variation	skewness	miniature end-plate potential		coefficient of variation	skewness
	frequency	amplitude			frequency	amplitude		
	s^{-1}	mV			s^{-1}	mV		
Control	2.04 ± 0.21	0.48 ± 0.03	0.32 ± 0.02	0.00 ± 0.02 (36,7)	5.90 ± 0.46	0.46 ± 0.07	0.33 ± 0.02	0.08 ± 0.02 (18.5)
Days after botulinisation								
1	0.55 ± 0.08	0.45 ± 0.06	0.69 ± 0.04	0.29 ± 0.02 (7,2)	0.60 ± 0.13	0.65 ± 0.16	0.68 ± 0.02	0.23 ± 0.03 (5,2)
2	0.30 ± 0.06	0.30 ± 0.01	0.60 ± 0.05	0.27 ± 0.05 (10,4)	0.42 ± 0.07	0.47 ± 0.04	3.64 ± 0.04	0.26 ± 0.05 (8,4)
3	0.48 ± 0.10	0.48 ± 0.07	0.70 ± 0.04	0.25 ± 0.04 (11,4)	0.72 ± 0.07	0.64 ± 0.13	0.68 ± 0.04	0.30 ± 0.04 (9,4)

glutinin complex was chromatographed on DEAE-Sephacel as before. A highly toxic protein appeared in the void volume followed by two very much less toxic peaks that were eluted with a salt gradient. The first peak contained about 18% of the applied neurotoxicity; it had a specific neurotoxicity of 37 mouse LD_{50} units/ng protein but lacked detectable haemagglutinin (Table 2). When analysed by electrophoretic and immunodiffusion techniques this material was identical to the pure neurotoxin except that it contained contaminants, including the 1.3×10^5 M_r component. In contrast, neurotoxin purified by procedure A was electrophoretically homogeneous under all conditions tested and the relative amount recovered was at least 2.5-fold higher than with procedure B (Table 2).

The second protein peak, which was eluted at 200 mM NaCl contained haemagglutinin activity (Table 2) that showed a 51-fold enrichment relative to starting material (Table 1). This purified haemagglutinin showed a precipitin line which coalesced with the equivalent line obtained with haemagglutinin-neurotoxin complex (Fig. 3).

The third protein peak eluted at 280 mM NaCl, had a pI value of 5.2 on polyacrylamide gel isoelectric focussing and showed no haemagglutinin activity and negligible neurotoxicity (Table 2). On electrophoretic and immunological analyses this protein corresponded to the 1.3×10^5-M_r component obtained with procedure A.

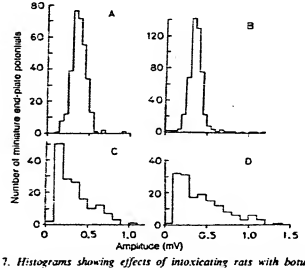

Fig. 7. *Histograms showing effects of intoxicating rats with botulinum neurotoxin on amplitude of muscle miniature end-plate potentials in the absence and presence of β-bungarotoxin.* Electrophysiological measurements were made as described in Materials and Methods on extensor digitorum longus muscle of control rats untreated (A) and within 14–45 min after addition *in vitro* of 0.14 μM β-bungarotoxin (B). Recordings are shown for muscle taken from rats injected 2 days previously with 60 pg pure neurotoxin before (C) and after (D) treatment with β-bungarotoxin *in vitro* as in (B)

Action of Botulinum Neurotoxin on Release of Acetylcholine at the Neuromuscular Junction

Within 15 min of applying the neurotoxin (4.7×10^3 mouse LD_{50} units/ml) there was a slight increase in indirectly elicited twitch tension lasting for approximately 20 min. After a further 90 min onset of near complete blockade was observed. Neither blockade nor increase in twitch tension was seen with heat-inactivated (100 °C for 3 min) toxin. Direct stimulation of the poisoned muscle or bath application of carbamoylcholine produced contraction, consistent with the neurotoxin acting presynaptically as demonstrated with another preparation of neurotoxin [7].

When this neurotoxin (5 mouse LD_{50} units; 60 pg protein) was injected into rat hind-leg muscle, it produced local paralysis within 24 h. Stimulation (square pulses of 0.1 ms duration, supramaximal voltage) of the nerve innervating the extensor digitorum longus muscle (1, 2 or 7 days after botulinisation) produced end-plate potentials of small amplitude (0.5–2 mV) with a large number of failures (S. Thesleff, personal communication).

In muscles from rats botulinised for 1–3 days, miniature end-plate potentials occurred with a significant reduction in frequency ($P < 0.005$) relative to controls (Table 3). Their mean amplitudes progressively decreased up to 2 days (significant reduction, $P < 0.0005$) after poisoning; at 3 days control values were restored (Table 3) and exceeded by the seventh day (data not shown). In botulinised muscles, the distributions of miniature end-plate potentials were skewed as compared with the 'bell shaped' distributions obtained in controls (Fig. 7A and C; significant increase in coefficient of variation and skewness, $P < 0.0005$, Table 3).

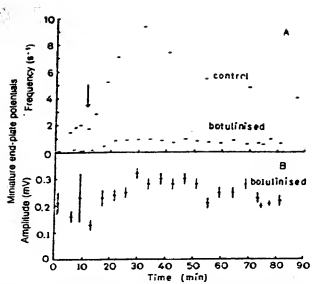

Fig. 8. *Time course of the effects of β-bungarotoxin on the frequency and amplitude of miniature end-plate potentials of muscle from normal and botulinised rats.* Continuous recordings of frequency (A) and amplitude (B) of miniature end-plate potentials were performed as described in Materials and Methods; measurements were made in the same fibre of botulinised muscle in (A) and (B). Botulinised rats were injected 3 days before the experiment with 60 pg botulinum neurotoxin. The arrow indicates the time of addition of 0.14 μM β-bungarotoxin in both (A) and (B). The end-plate of the botulinised muscle used had miniature end-plate potentials with unusually small amplitude. In (A) and (B) the length of horizontal bars represents periods of analyses; vertical bars represent standard error of each value

The effects of *in vitro* application of the presynaptically acting protein, β-bungarotoxin [22], were examined next. In controls this toxin produced a large, significant increase ($P < 0.0005$) in the frequency of miniature end-plate potentials after a short latent period (Fig. 8A, Table 3) [22]. Recording of the frequency in botulinised muscles showed that addition of β-bungarotoxin produced a much smaller increase (significant after 3 days $P < 0.05$) relative to controls (Fig. 8A, Table 3). In normal muscles it is notable that β-bungarotoxin did not significantly affect the mean amplitude (Table 3) of miniature end-plate potentials, while the amplitude distributions remained bell shaped (Fig. 7A and B). Similar treatment of botulinised muscles with β-bungarotoxin caused the mean amplitude of miniatures to increase (Table 3, Fig. 8B, significant increase, $P < 0.0005$, for 2-day-treated animals). The overall shape of the skew distribution of miniature amplitudes was not affected by the β-bungarotoxin (Fig. 7C and D) as was confirmed by the lack of significant change in skewness and coefficient of variation (Table 3). Thus, β-bungarotoxin scaled up all the amplitudes of miniatures in botulinised muscle, without otherwise affecting the shape of their distribution.

DISCUSSION

In this work reproducible, efficient methods were developed for the large-scale production of botulinum toxin and the purification to homogeneity of the neurotoxic protein. Solubilisation of acid-precipitated toxin has been attempted with water [2] and $CaCl_2$ solutions [14, 15] but in our hands the latter gave very variable and lower recoveries than with the phosphate buffer noted; this accords with published findings for type B toxin [23]. The specific neurotoxicity of the conjugate type A toxin obtained (25 mouse LD_{50} units/ng protein) is close to that (30–37 LD_{50} units/ng) obtained by others [15, 24] using similar methods. Gel filtration offers a more efficient and convenient alternative to lengthy crystallisation steps [24] for obtaining the major oligomeric form of the neurotoxin-haemagglutinin complex. However, it can be seen that the presence of multiple forms does not significantly lower the specific neurotoxicity or interfere with subsequent neurotoxin purification. In fact, they yielded homogeneous neurotoxin with the same molecular weight, pI and polypeptide composition but, of course, this does not preclude other intrinsic differences. Also, the presence of additional forms of toxin did not alter the patterns seen on immunodiffusion gels from that observed with the crystalline complex.

Affinity chromatography on immobilised *p*-aminophenyl β-D-thiogalactopyranoside is an excellent method for separation of haemagglutinin-free protein from heterogeneous preparations of complex but, as observed by Moberg and Sugiyama [5], fails to produce homogeneous neurotoxin. However, the use of a subsequent chromatographic step on DEAE-Sephacel removes the tightly associated contaminant which was shown to have a different molecular weight, subunit composition, immunoreactivity and very low neurotoxicity compared to the neurotoxin. Use of the latter step alone does not give complete removal of this 1.3×10^5 molecular weight contaminant and in addition, gives a 2.5-fold lower yield of the neurotoxin than the sequential affinity/ion-exchange chromatography method. The nature of the 1.3×10^5-M_r protein is unclear but its different properties noted above including being antigenically dissimilar from the neurotoxin suggests these are two distinct proteins.

The purified neurotoxin with a molecular weight of 1.4×10^5 is homogeneous by all criteria tested including dodecyl sulphate gel electrophoresis under non-reducing conditions, isoelectric focussing and immunodiffusion. This level of purity is essential for the preparation of a radiolabelled derivative and subsequent purification of a single radioactive species. Already we have prepared a highly radioactive iodinated derivative, which retained biological activity, and this is being effectively used for characterisation and localisation of its binding sites on nerve membranes [10]. The presence of two subunits ($M_r = 9.9 \times 10^4$ and 5.5×10^4) linked by disulphide bonds confirms previous findings [4]. Likewise, its specific neurotoxicity (83 mouse LD_{50} units/ng protein) differs little from the maximum values (186 units/ng) obtained for another preparation [4]. This is probably a negligible difference in view of the variation observed with the mouse toxicity assay and the fact that protein concentrations were measured differently.

As clearly demonstrated with impure neurotoxin complexes [19, 25], pure neurotoxin specifically and characteristically inhibited stimulated and spontaneous release of acetylcholine at the vertebrate neuromuscular junction. Our observations are similar to those reported for frog muscles treated with much higher doses of type D toxin [25] or type A neurotoxin [7]. Botulinum toxin appears to reduce transmitter release without seeming to affect calcium entry into nerve terminals or altering the filling of vesicles with acetylcholine [19, 26]. This suggests that it is the calcium triggered exocytosis step that is impaired by the toxin.

To help gain further insight into the mechanism of action of botulinum neurotoxin, we investigated whether β-bungarotoxin, which is thought to bind to a presynaptic site concerned with release, had its normal action on botulinised

muscle. In control muscle, during the time period investigated here, β-bungarotoxin alone gave a large increase in spontaneous release due to its phospholipase activity [22]. There was no change in amplitudes and distributions of miniatures suggesting that acetylcholine release continued to occur from the normal sites, albeit with higher frequency. In botulinised muscle, a small absolute increase in spontaneous release occurred together with a small increase in amplitudes of miniatures, while the amplitude distribution remained skewed (as was the case for botulinum toxin alone). We suggest that vesicle emptying by membranc fusion is impaired in botulinised muscle and this leads to less frequent miniatures. Following specific binding of β-bungarotoxin its intrinsic phospholipase activity, which potentiates its neurotoxicity, may aid vesicle emptying leading not only to a small increase in frequency, but also to a scaling up of all miniature end-plate potential amplitudes. The slow rise-times of miniatures observed in botulinised muscles are restored to normal values by β-bungarotoxin (unpublished). This result, together with the above observations on miniature amplitudes, indicates that the abnormal release seen after botulinisation occurs at sites in the active zones.

In a study of the interaction of these toxins, Chang and Huang [27] measured twitch tensions while crude botulinum haemagglutinin-neurotoxin complex and partially purified β-bungarotoxin were applied. They suggested that these toxins exhibit mutual antagonism of their inhibitory effects on release. Of course it was not feasible with their technique to measure the changes in transmitter release induced by β-bungarotoxin on botulinised muscle, as shown herein.

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